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19. *ABSTRACT*

Previous work by the principal investigators and others has shown that the major type of synaptic receptor for the major inhibitory neurotransmitter, the GABA-A receptor/chloride channel complex, is the target of numerous drugs and toxins. The GABA-A receptor function is directly potentiated by several categories of central nervous system depressants including benzodiazepines, barbiturates, steroid anesthetics, avermectin pesticides, and possibly ethanol. GABA-A receptor function is directly blocked by the GABA antagonist bicuculline, benzodiazepine inverse agonists, convulsant barbiturates, and a series of cyclic convulsant molecules like picrotoxin. These neuroexcitatory GABA blockers include pentylenetetrazol, chlorinated hydrocarbon insecticides like dieldrin and lindane, and the synthetic cage convulsants of Casida, such as t-butyl bicyclophosphorothionate (TBPS), one of the most toxic substances to mammals ever encountered.

We demonstrated that these convulsant drugs acted potently on GABA-A receptors in mammals and invertebrates, using a combination of electrophysiology and biochemistry. However, the differences in pharmacological profiles for GABAergic drugs between different animal species appeared important to define, such as how dangerous to non-target species are the currently used pesticides that act via the nervous system, and are there any potential new pesticides among the numerous GABAergic drugs active in mammals including man?

Using radioligand assays that we developed for sites on the GABA-A receptor complex, we were able to localize for the first time GABA-A receptors in the insect nervous system. This will be useful in understanding the physiology and toxicology of GABA. We also began studies on biochemical isolation of invertebrate GABA-A receptor proteins in hopes of determining their molecular structure.

A phylogenetic comparison of the GABA-A receptors was begun, and the subunit/gene composition in several animal species investigated. We identified the codfish as having a single polypeptide/gene for GABA-A receptors, as compared to 10-15 different subunits/genes in mammals. The codfish is thus closely related to the ancestral gene from which all the mammalian genes evolved. Structural and pharmacological comparisons of the different subtypes of GABA-A receptors in various species and in various regions of human brain are underway. This will help to define the specificity of the neurotransmitter and drug binding sites in the various receptors and should lead to the development of new useful drugs.

NEUROEXCITATORY DRUG RECEPTORS IN
MAMMALS AND INVERTEBRATES

Final Report

Richard W. Olsen and Thomas A. Miller

March 16, 1990

U.S. Army Research Office

Contract/Grant Number: DAAL 03-87-K-0020

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A. Statement of the Problem Studied

As described in the final report (11/30/86) for our previous contract with the same title (DAAG 29-83-K-0156), this project deals with comparative pharmacology of the $GABA_A$ inhibitory synaptic transmission in vertebrates and invertebrates, using a combination of biochemical, physiological, and anatomical approaches. We (1,2) and others (3) have described the interaction of a remarkable array of drugs with the mammalian brain $GABA_A$ receptor-chloride channel complex. These include many highly toxic convulsants and several important environmental pesticides, drugs which effect their actions through blocking of GABA receptor functions. The GABA receptor-chloride channel system is important in wide-spread regions of the mammalian nervous system and also wide-spread throughout the animal kingdom. We developed radioligand binding assays for GABA receptors ([^{3}H]muscimol; 4,5,23), and the associated chloride channels ([^{3}H]picrotoxin, 6; and [^{35}S]TBPS, 7) in invertebrates, contributed significantly to the development of such assays in vertebrates, e.g., (1,8,9), and studied numerous drugs for effects on these binding assays and on GABA receptor function assayed by tracer radiolabeled $^{36}Cl^-$ flux in mammalian brain slices (10,11) and by electrophysiological techniques in invertebrate tissues (12,13).

The objectives for the current contract period 1986-89 were: [A] to compare the GABA receptor proteins at the molecular level across animal species with the comparative pharmacological profile of drugs active on these species; and [B] to use the binding assays we had developed for the invertebrate GABA receptor chloride channel complex to: [i] describe the anatomical distribution of receptors in the insect nervous system for the first time, and [ii] to isolate the receptor proteins from invertebrate species for the first time, for comparative structure-function studies including molecular cloning.

B. Summary of the Most Important Results

1. Localization of GABA Receptors in Insect CNS

Conventional film autoradiography was used at the light microscopic level for the localization and quantification of γ-aminobutyric acid (GABA) receptors in the locust brain (*Schistocerca americana*). Localization of the receptor sites was achieved via binding with the receptor-ligand probe [^{3}H]muscimol (4). Frozen sections were cut and subsequently incubated either in 40 nM [^{3}H]muscimol or by coincubating sections with [^{3}H]muscimol and one of the following: GABA (50 μM), a receptor specific agonist (muscimol, 1 μM, or isoguvacine, 1 μM), an uptake inhibitor (nipecotic acid, 50 μM), or a noncompetitive channel modulator (avermectin B1a, 1 μM, or aldrin, 50 μM).

Through computer image enhancement and densitometric analysis of the optical density of [^{3}H]muscimol binding sites, the interaction of the above compounds with the putative GABA receptor was determined for various anatomical regions of the locust brain. By comparing the differently treated, but adjacent sections, GABA receptor distribution was quantitated and mapped (14). For this analysis, we employed an image analyzer purchased by Drs. Olsen and de Vellis under support of USARO.

Receptor sites were found distributed in the antennal lobes, central body, alpha-lobe and beta-lobe of the corpus pedunculatum, protocerebral bridge, and calyx as well as the optic lobe regions.

The GABA system is an important component of the insect central nervous system. Previous work has shown that both GABA concentrations and glutamic acid decarboxylase (GAD) specific activity exceeded by 10-fold that found in comparable vertebrate brain tissue (15).

The invertebrate tissues afford more accessible GABA sites and should be preferred for both neurochemical and receptor binding studies. Neuropharmacology combined with radiolabeled binding studies using computer-aided autoradiography will provide strong and direct evidence in substantiating that the GABA receptor in insects is a site of drug action. Through image enhancement and analysis, computer-acquired images of autoradiograms can now be quantified using standard binding procedures. Competitive binding studies can be performed on adjacent tissue sections *in situ* and receptors localized to specific anatomical regions (5).

The data indicate specific binding to GABA receptor sites. Serial sections are being analyzed by computer (16) to allow 3-dimensional reconstruction of the receptor distribution in the entire nervous system of locust and other insects. Work in progress includes similar autoradiography on [^{35}S]TBPS binding to the GABA receptor-associated chloride channels (7) in frozen unfixed tissue sections of insect ganglia.

2. Invertebrate GABA Receptor Neurochemistry

An undergraduate student from Bath University, United Kingdom, Mr. Damian Cotton, working under our collaborator Dr. George Lunt, came to Dr. Olsen's laboratory at UCLA (April-September, 1988) to work on crayfish and insect GABA receptors. Mr. Cotton found that GABA receptors are present in high density in abdominal muscles of crayfish (living outdoors) only during warm summer months. He successfully managed to solubilize with mild detergent the crayfish muscle GABA receptor complex, preparatory to its biochemical purification. We have not had anyone continuing the project recently, but plan to return to it soon. However, we may actually obtain the invertebrate receptor protein sequences more rapidly by cloning, using the vertebrate cDNA (17-19) as probes.

In addition, Mr. Cotton learned our housefly head membrane preparations and assays of [^{3}H]muscimol and [^{35}S]TBPS binding to teach them to the Lunt laboratory in Bath, preparatory to measuring their molecular weights (target size) by irradiation inactivation, in collaboration with Dr. Mogens Nielsen in Roskilde, Denmark. Unfortunately, ill health prevented Mr. Cotton from completing this project, but we (Olsen, Lunt, Nielsen) plan to continue the work soon.

<u>Comparative biochemistry of GABA receptor protein</u>: Lynn Deng, graduate student in Dr. Olsen's laboratory at UCLA, has been working on the purification, photoaffinity labeling, and subunit composition of the GABA/benzodiazepine receptor from codfish. In collaboration with Dr. Mogens Nielsen of Denmark, we found codfish brain [^{3}H]flunitrazepam binding protein to have a molecular weight of 58 kiloDaltons on SDS-PAGE (20). Then the protein was purified by benzodiazepine affinity chromatography as we described

for rat receptor (21): a single stained band was observed on SDS-PAGE at 58 kD. Photoaffinity labeling with both [^{3}H]flunitrazepam and [^{3}H]muscimol resulted in a single radioactive peptide band corresponding to the single stained band at 58 kD (22). The purified codfish receptor was subjected to 2-dimensional gel electrophoresis and showed only 1 spot. This preparation gave a specific activity of about 1000 pmol of [^{3}H]muscimol and 500 pmol of [^{3}H]flunitrazepam binding per mg protein. The latter was enhanced by GABA in the assays and showed central benzodiazepine receptor specificity. Thus, the codfish receptor appears to consist of a single subunit, in contrast to mammalian species which have two subunits of different size but homologous sequence. The mammalian subunits of the GABA receptor may have evolved from a common ancestral gene, while the codfish protein still is coded for by the single ancestral gene (Deng, Nielsen and Olsen, manuscript in prepatation). It will be of interest also to examine the subunit composition of invertebrate GABA receptors.

Current studies include attempts to measure the native molecular weight of the codfish receptor, and the question of whether barbiturate, picrotoxin, and steroid receptor sites are present on the complex, as they are in mammals. We will also attempt to isolate fresh mRNA from codfish brain for the construction of a cDNA library and cloning of the gene(s) for codfish GABA receptor using mammalian cDNA probes and the polymerase chain reaction. In addition, we have prepared receptor protein (~1 mg) from 200 g of codfish brain for two approaches to molecular structure: [i] sequencing of ligand binding active sites using proteolytic fragmentation of photoaffinity labeled subunits as we have done on mammalian protein; [ii] attempt to produce a water-soluble large fragment containing the GABA binding site, presumably in the N-terminal 200 residues, using [^{3}H]muscimol photolabeled protein as starting material. Such a fragment might be suitable for crystallization and X-ray structural work, especially since the codfish protein, unlike the mammalian protein, appears to be a <u>homo</u>-oligomer. Note that <u>no</u> neurotransmitter receptor even in part has been crystallized so this active site fragment is a potential solution both to the overall structure and to accurate ligand binding site information.

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